Evidence Report:

Risk of Adverse Health Effects Due to Host-Microorganism Interactions

Current Contributing Authors:

C. Mark Ott, PhD	NASA Johnson Space Center, Houston, TX
Audrie Colorado, PhD	KBR, Houston, TX

Previous Contributing Authors:

Cherie Oubre, PhD	NASA Johnson Space Center, Houston, TX
Sarah Wallace, PhD	NASA Johnson Space Center, Houston, TX
Satish Mehta, PhD	JES Tech, Houston, TX
Duane Pierson, PhD	NASA Johnson Space Center, Houston, TX
Anuran Chatterjee, PhD	NASA Ames Research Center, Moffett Field, CA
Sharmila Bhattacharya, PhD	NASA Ames Research Center, Moffett Field, CA

Human Research Program
Human Health Countermeasures Element

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LIST OF ACRONYMS

CMV – Cytomegalovirus

EBV – Epstein-Barr virus

GAP – Group Activation Pack

HRP – Human Research Program

HSL – Homoserine Lactone

IOM – Institute of Medicine

ISS – International Space Station

MEED – Microbial Ecology Evaluation Device

NASA – National Aeronautics and Space Administration

QS – Quorum Sensing

RNA – Ribonucleic Acid

RPM – Random Positioning Machine

RWV – Rotating Wall Vessel

STS – Space Transportation System

VZV – Varicella-Zoster Virus

RISK STATUS: Active

EXECUTIVE SUMMARY

While preventive measures limit the presence of many medically significant microorganisms during spaceflight missions, microbial infection of astronauts cannot be completely prevented and does occur [1-3], despite stringent vehicle cleaning and monitoring, as well as a quarantine of astronauts prior to flight. The reason behind this persistence of infection may have several causes, including unexpected microbial responses to spaceflight culture, which have been observed in spaceflight and spaceflight analogue experiments over the past 55 years [4,5]. While the stimulus/stimuli and mechanism(s) behind those responses are unclear, the operational relevance of these unexpected microbial responses was emphasized by the results of an experiment aboard STS-115 in 2007 which demonstrated that spaceflight culture of the enteric pathogen *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) increased its virulence in a murine model of infection [6]. The experiment was reproduced in 2008 aboard STS-123 confirming this finding [7]. In response to this evidence, the Institute of Medicine (currently the National Academy of Medicine) recommended that the National Aeronautics and Space Administration (NASA) investigate this risk and its potential impact on crew health during spaceflight. NASA assigned this risk to the Human Research Program (HRP) to better understand how spaceflight associated host-microorganism factors could alter the risk to astronaut health and performance during a mission. Importantly, while spaceflight and spaceflight analogue studies have continued since the initiation of this risk, the cause of these changes is unclear, leaving a major knowledge gap that limits risk assessments and countermeasure development.

As the interaction between the astronaut, the microorganism, and the space habitat environment (including life support systems) is highly interactive, the influence of each has been included for evaluation of this risk assessment. Accordingly, the risk contribution of spaceflight food, immune system function, the vehicle environment, and other spaceflight associated factors (*e.g.*, radiation or celestial dust exposure) are included, as they may contribute to the overall crew health risk. In addition, the threat to the crew in this this risk extends to problems that may result from contamination of the vehicle habitat and systems (*e.g.*, potable water system). While the environmental microbiome of spaceflight habitats is well understood, a more evidence of previously uninvestigated niches (*e.g.*, plant growth facilities) is still needed.

Collectively, further research is warranted to decrease the uncertainty in the risk assessment process and to understand the contribution of spaceflight-specific factors to determine how spaceflight alters risk associated with host-microorganism interactions.

SECTION I: EVIDENCE

Introduction

In 2008, the Institute of Medicine (IOM) of the National Academies (currently the National Academy of Medicine) reviewed the Human Research Program Evidence Book of the "Risk of Crew Adverse Health Event Due to Altered Immune Response." The IOM cited research from a flight experiment by Nickerson and colleagues aboard STS-115, which indicated that the enteric pathogen, *S.* Typhimurium had become more virulent when cultured during spaceflight [6]. The IOM recommended NASA "Develop evidence books on additional risks, including alterations in microbe and host interactions…" In November 2008, a risk entitled, "Risk of Adverse Health Effects Due to Alterations in Host-Microorganism Interactions," was added to the Human Research Program's Integrated Research Plan to determine the likelihood and consequences of alterations in microbial interactions with the crew and their environment that could impact their health and performance.

Enclosed habitats, including spacecraft, contain microorganisms of human and environmental origin, including opportunistic and possibly obligate pathogens. Even with preventative measures like the Crew Health Stabilization Program [8], microbiologically-related risk to astronaut health from microorganisms is well documented [1-3]. While often difficult to diagnose, evidence from Space Shuttle crewmembers suggest past occurrence of fungal infection, flu-like illness, urinary tract infection, aphthous stomatitis, viral gastroenteritis, subcutaneous skin infection, and other viral disease [3].

Microbiological requirements to protect crew health, life support systems, and vehicle integrity during spaceflight have been subjectively extrapolated from terrestrial requirements based upon limited environmental and clinical monitoring results. In many cases they do not include spaceflight-specific contributing effects, such crew exposure to radiation, diminished immune status, or inhalation of celestial dust. At the heart of this risk is defining the potential of disease occurrence that would exceeds terrestrial expectation and the countermeasures necessary to mitigate that risk.

For this report, virulence refers to the ability to manifest and severity of disease, which requires a host that can display the symptoms and sequalae of that disease. Accordingly, to evaluate changes in the virulence of pathogens during spaceflight, animal models of microbial infection are required based on the need for complex models with inflammatory and pathological characteristics that closely resemble human responses during infection. Animal models of infection have demonstrated excellent reproducibility of the pathological response which can be measured under varying nutritional, pharmaceutical, and environmental conditions, supporting the development of effective countermeasures. The use of animal models has also benefitted our understanding of the potential risk of microbial infection through investigations of alteration in the immune system during spaceflight [9-13]. Likewise, ground-based hind-limb unloading models have been used to investigate some of the effects of spaceflight on microbial infection [14,15]. Appropriate animal models of infection have not been limited to murine models, as demonstrated by the successful use of *Drosophila melanogaster* (fruit fly) in both ground-based and spaceflight experiments [16,17].

While many alterations in microbial responses to spaceflight culture have been well-documented over the past 55 years [4,5,18,19], this Evidence Report focuses only on those responses that substantially impact this HRP *Risk of Adverse Health Effects Due to Host-Microorganism Interactions*. Efforts on this risk integrates with other disciplines to gather information and determine the overall impact to the astronaut. Evidence that is pertinent to this Risk is described in greater detail in other evidence reports, including *Risk of Adverse Health Event Due to Altered Immune Response, Risk of Adverse Outcomes Due to Inadequate Human Systems Integration Architecture, Risk of Performance Decrement and Crew Illness Due to Inadequate Food and Nutrition, Risk of Adverse Cognitive or Behavioral Conditions and Psychiatric Disorders*, and *Risk of Performance and Behavioral Health Decrements Due to Inadequate Cooperation, Coordination, Communication, and Psychosocial Adaptation within a Team*.

Evidence

Human Spaceflight

Microbially-associated disease has been documented multiple times during spaceflight missions. Prior to the implementation of the Health Stabilization Program during the Apollo Program, 57% of the prime crews experienced some illness, including upper respiratory infections, viral gastroenteritis, rhinitis/pharyngitis and one rubella exposure [8,20]. Perhaps the most visible incidence of infection during spaceflight occurred during Apollo 13 in which a crewmember suffered a urinary tract infection caused by *Pseudomonas aeruginosa* [20]. Treatment with antibiotics (furadantin and pyridium) during the mission were ineffective.

A survey of disease during the Space Shuttle Program (STS-1 through STS-89) indicated that infectious disease accounted for 1.4% of all medical events (not including skin and subcutaneous tissue) [2]. Notable among the infections during the Shuttle Program was a thoracic zoster infection two days prior to launch [21]. Through 2016, infectious disease and allergic symptom rates on ISS have been estimated at 3.4 events/flight year [1].

Spaceflight associated alterations to astronaut microbiome are also a pertinent consideration for this risk. Early studies of the cosmonaut microbiome provided excellent foundational evidence [22-26], including previous evaluations of *Bifidobacterium* in cosmonauts by Goncharova, *et al.*, which identified preflight decreases in bifidobacteria and alterations in acid formation during flight [27]. Recent advances in genomic capabilities have provided an opportunity for greater translation of human microbiome data [28]. In the most comprehensive genomic study to date into alterations in astronaut microbiomes, Voorhies, *et al*. evaluated the microbiomes of nine crewmembers over the course of their mission. Samples included skin (forehead, forearm), nose, tongue, and feces for taxonomic analysis, as well as saliva and blood for immunological evaluation [29]. Overall, the intestinal microbiota became more similar over time in flight, which could be attributed to their common diet. Interestingly, the skin microbiome was also altered, which the authors speculated may be the result of changes in moisture and pH and/or astronauts' personal hygiene habits. Another notable study of the astronaut microbiome was during the "Twins Study" [30]. The study supported previous observations indicating spaceflight-associated alterations in the astronaut microbiome; however, the limited dataset limited conclusions. While published data (and the corresponding sample size) remain small, new studies are incorporating microbiome components

into their analyses, which should provide a better understanding of an interrelation of human physiological conditions, including those associated with immune function, nutrition and behavior, especially as they relate to the gut-brain axis [31].

Extensive research has shown that astronauts are also at risk of reactivation of latent viruses during spaceflight [21,32-35], which could lead to disease. Astronauts shed Epstein-Barr virus (EBV) in saliva before, during, and after spaceflight. Frequency of shedding in astronauts can be several times higher than control subjects, especially during flight [35]. Varicella Zoster Virus (VZV) is not commonly identified in the saliva of astronauts before flight or in matching ground-control subjects; however, VZV was shed in ~50% of crewmembers during flight and continued up to ~5 days after landing [36]. Aboard the ISS, approximately 60% of astronauts shed VZV during the flight phase and some can shed the virus at least 30 days after flight [37]. A few cases of zoster have occurred either before, during, or after spaceflight. Mehta and Pierson showed that 47% of Space Shuttle astronauts shed cytomegalovirus (CMV) in urine during spaceflight and continued for two weeks after flight. Whereas, less than 1% of control subjects shed CMV [33]. Follow-up studies showed that 73% of ISS astronauts shed CMV and shedding continued for 30 days after landing. In one study of 71 astronauts, 77% were seropositive. The cause of the viral reactivation has not been completely determined; however, evidence suggests that one contributing factor is stress. Glaser, et al. demonstrated a relationship between chronically stressed individuals and cellular immunity and increased antibodies to EBV [38]. Studies have also linked psychological stress with onset and severity of infectious mononucleosis [39].

Microorganism Spaceflight

Host-Pathogen Studies. While the highest fidelity spaceflight experiments investigating host-pathogen interactions would be those with both microbial growth and infection occurring during spaceflight, this approach is extremely difficult, as it is limited by biosafety considerations and inability to infect an acceptable number of human-surrogate animals with controlled doses of the pathogen. To address the inherent limitations, most spaceflight experiments investigating host-pathogen interaction have focused on the exposure/growth of microorganisms in the spaceflight environment and returning those cultures back to earth. For example, in one of the first reports of its kind, cultures of *Saccharomyces cerevisiae* collected from the Microbial Ecology Evaluation Device (MEED) aboard Apollo 16 displayed a higher recovery rate from dermal lesions (induced by injection) than those recovered from ground controls [40]. It is important to note that these cultures were exposed to ultraviolet radiation during the mission and were not immediately tested after landing, however these findings suggested the potential of clinical implications associated with microbial growth in the spaceflight environment.

The earliest investigations into alterations in the virulence and global gene expression associated with growth in the spaceflight environment focused on the enteric pathogen *S.* Typhimurium, a leading cause of foodborne illness [6,7]. *Salmonella* species have been recovered from the Space Shuttle [41] and in spaceflight food destined for the ISS [42], and thus are relevant threats to crew health. In separate experiment aboard STS-115 and STS-123, spaceflight-cultured *Salmonella* exhibited increased virulence in a murine infection model compared to control cultures grown on Earth [6,7]. Specifically, cultures grown during STS-115 in a Lennox Broth medium during flight caused a reduced time-to-death, increased percent mortality, and displayed a 2.7-fold lower LD_{50} (lethal dose required to kill 50% of the mice) in a murine infection model when compared to

inoculation with ground-control cultures [6] (**Figure 1**). Similar patterns of increased virulence were observed when the experiment was repeated aboard STS-123, as the LD_{50} decreased 6.9-fold [7]. Transcriptomic and proteomic profiling revealed differential regulation of 167 genes and 73 proteins. Interestingly, key genes known to be important for *Salmonella* virulence were not regulated as expected, suggesting novel mechanisms for the observed spaceflight-associated virulence phenotype [6]. In addition, spaceflight-induced increases in *Salmonella* virulence were shown to be regulated by media ion/salt concentration, as modulation of these salt concentrations could be used to turn off the increased virulence [7]. Furthermore, *Salmonella* biofilms were uniquely formed in spaceflight conditions and not in ground controls [6]. Importantly, the evolutionarily conserved RNA chaperone protein, Hfq, was identified as a global regulator of the *S.* Typhimurium response to spaceflight culture [6].

Figure 1. Astronaut Dominic Gorie manually activates the Group Activation Pack (GAP) hardware containing S. Typhimurium aboard STS-123 to better understand bacterial responses to the spaceflight environment.*Image: NASA*

The spaceflight experiments with *S.* Typhimurium produced several key findings including: (1) the experiment clearly indicated alterations in the expected dose-response curves with implications for the microbial risk assessment of infection potential of the crew during a mission; (2) the experiment provided the first insight into a molecular mechanism behind the alterations of microorganisms during spaceflight culture; and (3) the virulence and gene expression results from the spaceflight experiment paralleled the trends observed with spaceflight analogue bioreactors [43], supporting the Rotating Wall Vessel (RWV) bioreactor as an indicator of potential microbial alterations during spaceflight.

Subsequent spaceflight studies showed that *P. aeruginosa* also responded to spaceflight conditions through differential regulation of 167 genes and 28 proteins, with Hfq as a global transcriptional regulator, identifying the first spaceflight-induced regulator acting across bacterial species [44]. This shared regulation may indicate that mechanical stimuli, like low fluid shear forces experienced by microbial pathogens in both the quiescent microgravity environment of spaceflight and on Earth during their natural life cycles, including in the infected host [4,45-49], may pre-adapt bacteria to be "hardwired" to respond to the microgravity environment. Recently, a second bacterial pathogen, *Serratia marcescens,* was also shown to exhibit increased virulence during spaceflight culture [17].

Pathogenic yeast have not been as extensively study compared to bacteria. However, pertinent to this risk, the response of *Candida albicans* to growth in the spaceflight environment was investigated [50]. The experiment identified 452 differentially regulated genes compared to ground controls, including genes associated with cell aggregation, oxidative stress resistance, antifungal resistance as well as the induction of ABC transporters. Random budding patterns (as opposed to bipolar budding normally expected on earth) were observed. Virulence studies with a murine model were performed, but no statistically significant alterations in virulence were observed.

As mentioned previously, infection of a host in spaceflight is challenging. However, a recent spaceflight study investigated the transcriptomic response of epithelial tissue culture (HT-29) and *S.* Typhimurium during infection when both were in the spaceflight environment [51]. While the sample size was relatively small, this investigation suggested that spaceflight significantly altered the transcriptional and proteomic profiles of both uninfected and infected host cells, inducing unique transcriptional and proteomic responses not observed in the identical ground controls. Trends observed between infected flight and ground samples were consistent with a heightened response of host cells to infection with *Salmonella* during spaceflight. Other models enabling a greater sample size are being investigated. Virulence studies using the nematode, *Caenorhabditis elegans*, as a human surrogate model of infection with *S.* Typhimurium have recently been completed aboard the ISS. The results of the experiment, designated as Micro-5, are being tracked for future inclusion in this report.

Other Pertinent Spaceflight Studies. The primary post-infection countermeasure during spaceflight is the use of antibiotics; however, several spaceflight experiments have provided evidence suggesting possible alterations in antibiotic resistance when microorganisms are cultured during spaceflight. During the Cytos 2 experiment aboard Salyut 7 in 1982, the minimum inhibitory concentration of oxacillin, chloramphenicol, and erythromycin for *Staphylococcus aureus* and of colistin and kanamycin for *Escherichia coli* were compared to those of ground controls [52,53]. These early results indicated an increased resistance of both *S. aureus* and *E. coli* to all antibiotics used in this experiment [52,53]. Conversely, spaceflight experiments culturing *E. coli* during STS-69 and STS-73 suggested gentamicin on agar slants that were flown was as effective as and possibly more effective than the antibiotic on ground-based control cultures [54]. In 1999, Juergensmeyer *et al*. observed both increased sensitivity and resistance by cultures of *S. aureus, P. aeruginosa, Bacillus subtilis*, and *E. coli* that had been re-grown after having been on the Mir space station for 4 months [55]. While some of these experiments suggest the possibility of spaceflight-associated changes in microbial response to antibiotics, the evidence is not adequate nor consistent enough to be predictive about the actual microbial response either *in vitro* or during exposure in a human host.

The study of biofilm formation during spaceflight is also pertinent to crew health risk, and the opportunistic pathogen, *P. aeruginosa*, was investigated in an early experiment, which confirmed its ability to form biofilms during spaceflight [56]. In a separate set of spaceflight experiments, Kim *et al*. investigated biofilm architecture of *P. aeruginosa* during spaceflight [57]. This research team found that the biofilm architecture was substantially different compared to Earth-grown controls, describing the biofilms as having a "column and canopy" structure that had not previously been reported. While the full implications of this finding are yet to be determined, it may have an impact on the control of biofilms in medical and environmental scenarios.

While many spaceflight experiments have been performed to assess the effects of this unique environment on microorganisms, there are several factors that complicate the evaluation and comparison of the resulting data. Some of these confounding elements include (a) the wide variety of organisms that have been studied, including motile versus non-motile bacteria; (b) the different spaceflight parameters that have been used (e.g., differences in lengths of missions, sample handling – fixed or frozen, in-flight centrifuged 1 x g controls versus ground 1 x g controls); and (c) differences in growth media used (e.g., minimal versus rich media or liquid versus solid media). While these factors complicate inter-study comparisons and conclusions, the overall indication is

that growth and infection in the spaceflight environment can be altered from the expected phenotype and function observed during traditional terrestrial experiments.

Microorganism Terrestrial

While spaceflight is the ultimate platform for performing experiments to determine alterations in microbial responses and host-pathogen interactions, spaceflight research is constrained by high costs, inconsistent flight availability, minimal in-flight analytical equipment, as well as limitations in power usage, payload weight and volume, and crew time. Thus, ground-based analogues have been developed to evaluate alterations in microbial responses to these conditions [4,58]. These analogues do not remove gravity from the system, but instead develop an environment that reflects many of the secondary effects observed in microgravity (decreased mass transfer, lower fluid shear, etc.).

Figure 2. Rotating wall vessel (RWV) bioreactor developed by NASA and used during ground-based microbiology experiments. Image: NASA

Most analogue systems rely on the continuous sedimentation of microbial cultures in a growth medium. The simplest system is the clinostat, which is a cylindrical tube completely filled with media (no bubbles, i.e., "zero headspace"), that is rotated perpendicular to the gravitational force vector [59]. Likewise, a more complex system designed by NASA, the RWV, has been used extensively since the mid-1990s (**Figure 2**). The RWV is also an optimized form of suspension culture, which consists of a hollow disk or cylinder that is completely filled with medium and rotates on an axis perpendicular to the gravitational force vector. Under these culture conditions, the cells are maintained in suspension as the RWV is rotated and a sustained low-shear environment for cell growth is achieved [4]. Exchange of nutrients and localized "mixing" of the microenvironment is facilitated by the constant falling of the cells through the local fluid environment and the gentle rotation of the culture medium. Unlike the clinostat, a gas-permeable membrane on one side of the RWV allows constant air exchange during growth.

Other microbial culture spaceflight analogues have been reported, such as the random positioning machine (RPM) and the use of diamagnetic levitation [60-62]. The RPM also suspends microorganisms in growth media; however, this suspension is maintained by randomly adjusting the movement of the bioreactor. Diamagnetic levitation relies on a strong magnetic field to levitate microbial cultures, and thus reproduce aspects of microgravity. As with all spaceflight analogues, the fidelity of these and other culture devices to reproduce culture during spaceflight is not completely known as the stimulus/stimuli and mechanism(s) driving the alterations in microbial response are unclear.

Spaceflight analogue systems have been shown to both (a) provide indications of spaceflight responses prior to spaceflight and (b) enable follow-up experimentation to provide insight into the spaceflight experiment. Evidence of these benefits is demonstrated by comparing responses of S.

Typhimurium cultured in the RWV and the same organism cultured during spaceflight [6,7,43]. Specifically, when S. Typhimurium was cultured in the RWV, the LD_{50} decreased 5.9-fold compared to reoriented controls, which provided an excellent indicator of a similar trend observed with *S.* Typhimurium grown on both STS-115 (2.9-fold decrease) and STS-123 (6.9-fold decrease) when compared to ground controls [6,7,43]. Likewise, similar trends in gene expression and regulation were also observed, as RWV-cultured *S.* Typhimurium displayed 163 genes with statistically significant changes in the level of expression, as compared to spaceflight culture (167 differentially regulated genes) [6,63]. While certain similar characteristics, such as *hfq* downregulation, were characteristic of both spaceflight and spaceflight analogue culture, all of the identified genes did not differentially express in the same fashion. This difference reinforced the limitations of analogue systems and need of spaceflight experiments to confirm phenotypic and molecular-genetic alterations that occur during spaceflight.

Terrestrial RWV studies with *S.* Typhimurium indicated several phenotypic changes that provided insight into the alteration in microbial characteristics that increased virulence. S. Typhimurium grown in the RWV also displayed altered stress responses and survival in macrophage cells compared to control cultures [43,63]. A comparison of microarray data from the RWV and control cultures indicated 163 differentially expressed genes distributed throughout the chromosome, representing functionally diverse groups including transcriptional regulators, virulence factors, lipopolysaccharide biosynthetic enzymes, iron-utilization enzymes, and proteins of unknown function [63]. These findings evaluated the strain, S. Typhimurium X3339. Other studies have investigated other strains of S. Typhimurium in the RWV [64], including a unique variant, S. Typhimurium 313 strain D23580 [65]. The D23580 strain is classified as S. Typhimurium; however, it is a highly virulent, multidrug resistant strain that infects through the blood system similar to *Salmonella enterica* serovar Typhi [65]. Interestingly, growth in the RWV increased the LD_{50} of D23580 compared to controls, reinforcing that the spaceflight analogue environment may actually induce a less virulent strain in some potential pathogens. This type of response was also observed in cultures of *S. aureus* grown in the RWV, which decreased carotenoid production and increased exopolysaccharide levels, suggesting a less virulent phenotype [66]. Taken together, these results suggest the stimulus/stimuli from growth in spaceflight analogue environments induce different responses based upon the benefit to individual species.

Characteristics of various strains of *E. coli* cultured in the RWV have been investigated [67-72]. Cultures of *E. coli* AMS6 in minimal media demonstrated an increased resistance to acid and osmotic stress in response to the low-shear conditions [69]. Interestingly, culture of this strain in the RWV displayed significantly higher biofilm production on glass microcarrier beads placed in the reactor [70]. Investigation of the response of adherent-invasive *E. coli* O83:H1 to culture in the RWV indicated this organism did not change growth, acid or osmotic resistance; however, it did display an increased resistance to thermal and oxidative stress in minimal media [73]. Interestingly, low-shear cultured *E. coli* O83:H1 displayed increased adherence to epithelial cells although invasion rates were unchanged as compared to controls [73].

Several RWV studies have also investigated the response of *P. aeruginosa* to growth in this environment [74,75]. *P. aeruginosa* cultured in the RWV displayed distinct changes in its biofilm architecture compared to controls, which could impact its virulence and antibiotic resistance [74]. In addition, RWV culture of *P. aeruginosa* appears to influence the *rhl* N-butanoyl-L-homoserine

lactone (C4-HSL) directed quorum sensing (QS) system, increasing the production of rhamnolipids, and potentially having an impact on the virulence of the organism [74]. Analysis of gene expression data also identified a role for the global regulatory protein, Hfq, as seen in *S.* Typhimurium [75].

Other organisms beyond gram-negative pathogens have been evaluated using the RWV. The response of *S. aureus* to RWV culture has been the most thoroughly studied among Gram-positive microorganisms [66,76-78]. Interestingly, while gene expression appears to be regulated by Hfq [66], as seen with *S.* Typhimurium and *P. aeruginosa*, virulence characteristics, such as staphyloxanthin production and hemolytic activity appear to be repressed [66,76]. Culture of *Streptococcus pneumoniae* in the RWV has also been studied, as 41 genes were reported to be differentially regulated [79]. The pathogenic yeast *C. albicans* displayed random budding patterns and enhanced filamentous growth when cultured in the RWV, suggesting a more pathogenic phenotype [80].

Computer-based models

Even with the semi-closed environment of the ISS, aligning environmental microbiological data with the incidence of disease is challenging due to the limited number of samples and sampling sessions. The use of machine learning techniques is currently being investigated in Translational Research Institute for Space Health (TRISH) and HRP studies. Using ISS environmental data, the foundation and approach developed during the TRISH study, will be compare with clinical data from astronauts in the HRP study. If successful, additional factors will be incorporated into prospective studies in the future, including crew immunological status, spaceflight-induced alterations in microbial virulence, and the impact of plants and rodents on the environment. This integrative approach will improve future decision making on countermeasures to prevent loss of crew or loss of crew performance due to adverse health events.

SECTION II: RISK IN CONTEXT OF EXPLORATION OPERATIONAL SCENARIOS

Microbiological operational activities are designed to mitigate the risk of microbially-associated disease (e.g., infection, allergic type response) by limiting crew exposure to opportunistic and obligate pathogens. When possible, risk assessments reflect the general guidelines described in the federal interagency document USDA/FSIS/2012-001 and EPA/100/J12/001, which outlines a process including hazard identification, evaluation of the dose-response of those agents, and the crew exposure to those agents [81]. The routes of infection include spaceflight environments (e.g., vehicle air and surfaces), spaceflight foods and potable water, cargo, payloads, and crewmembers.

Figure 3. Astronaut performing routine microbiology monitoring during spaceflight. Image: NASA

Stringent microbiological monitoring of spacecraft (**Figure 3**) has been performedoperationally aboard NASA spacecraft throughout the human spaceflight program [19,82,83]. The current microbial requirements for environmental samples were refined based on a series of forums with input from experts from industry, government and academia [84]. The monitoring regimen includes sampling vehicle air and surfaces, potable water, cargo payloads, and spaceflight foods. Experimental payloads are reviewed and assigned a biosafety level. Additional spaceflight experiments have also provided greater detailed information by investigating specific environmental niches aboard spacecraft or using alternative methodologies beyond the culture-based isolation historically used [85,86].

Currently, microbial enumeration of environmental samples is performed during space flight operations and samples are returned to the ground for microbial identification [82]. Generally, the data indicate that the environmental microbiome of the ISS and other spacecraft reflect the same microorganisms that populate a human home [87-89]. On ISS the potable water, air, and surfaces to which the crew are exposed are free of obligate pathogens; however, opportunistic pathogens such as *P. aeruginosa*, *Stenotrophomonas maltophilia*, and *S. aureus* are not uncommon [82,90]. In addition, identification of microorganisms collected from free-floating water behind panels indicated several potentially medically significant organisms not commonly isolated during standard operational monitoring, including *Legionella* species, and *Serratia marcescens* (*S. marcescens*), and *E. coli* [91]. Further microscopic examination of these samples revealed the presence of amoeba resembling *Acanthamoeba* or *Hartmanella* species and ciliated protozoa resembling *Stylonychia* species [91].

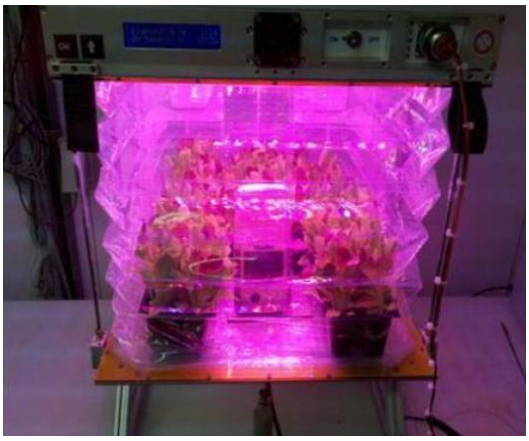


Figure 4. "Veggie" plant growth system used to grow pick-and-eat crops.

Spaceflight food is currently provided for missions in a shelf stable form for storage at ambient temperature [92]. As such, microbiological contamination control, including stringent microbial monitoring, is maintained. While the incidence of contamination is low, preflight analyses of food samples have indicated the presence of organisms such as *S.* Typhimurium, *S. aureus*, *Enterobacter cloacae* and *Cronobacter sakazakii* (unpublished data). Contaminated lots are removed before shipment for flight; however, the risk of food poisoning remains. Spaceflight missions on ISS provide food with potentially high levels of microorganisms, such as freshly grown crops or foods with probiotic organisms to promote astronaut health. Risk assessments and requirements to enable consumption of these foods are evaluated and set on a case-by-case basis (**Figure 4**).

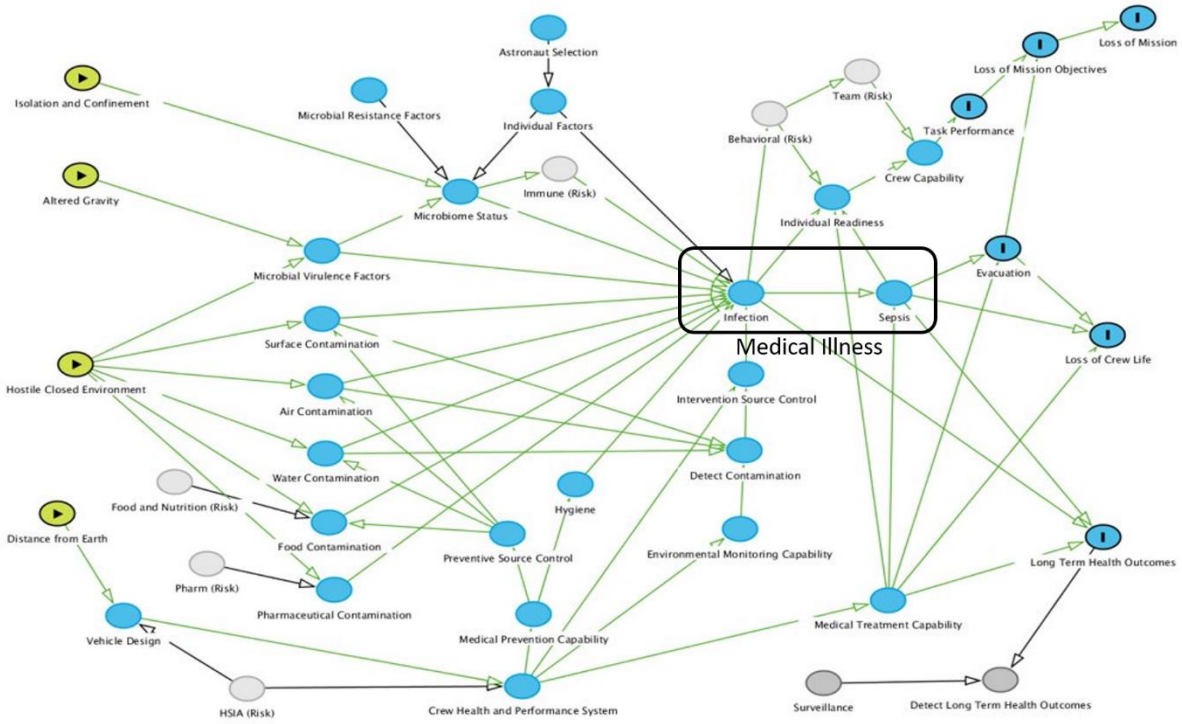
For spaceflight missions, the primary source of microorganisms is the crew. Selected preflight microbiological monitoring is performed prior to launch, with testing based on the mission design. One key aspect of preflight operations is NASA's Flight Crew Health Stabilization Program, which was established during the Apollo Program in response to problems with incidences of infectious illness [8]. The focus of the program involves reducing the exposure of flight crews to groups and individuals that are at high risk of harboring infectious disease (e.g., large crowds, small children) beginning approximately 10 to 14 days before launch.

Microbiological risk assessments and requirements aboard spacecraft are heavily integrated with life support system design and function. In general, microbiological mitigation techniques that can be engineered into the design of the vehicle and its systems is much more resource efficient than crew operational activities.

Overall, the current mitigation countermeasures used to minimize infectious and other microbially-induced disease are exceptionally effective. However, disease events continue to occur, and gaps in our knowledge create uncertainty in the overall risk assessment in determining both the incidence rate and the expected severity of infection.

SECTION III: DAG REVIEW and INTEGRATION WITH OTHER RISKS

DAG Review: [Pending Final Approval]



- The **Microhost risk** centers around the possibility for microbial contamination leading to Infections that if left inadequately treated could become sepsis. Both **Infections** and **Sepsis** can lead to deterioration of **Individual Readiness** and **Crew Capability** which affects **Task Performance**, likelihood of **Evacuation** for medical reasons, and in severe cases can contribute to **Loss of Crew Life**. They can also lead to **Long Term Health Outcomes** if inadequately treated and post-mission/career **Surveillance** enables **Detection of Long Term Outcomes** to understand the magnitude of the problem.
- The cause of infections can come from various sources:
  - Microbial Virulence Factors – evidence that the virulence of certain microbes changes in response to spaceflight environment.
  - This may lead to an increased risk of infections
  - Can indirectly lead to infections through changes in Microbiome Status
  - Immune (Risk) - the strength of the immune system determines how well individuals fight off infections
  - Surface Contamination - microbes on surfaces are found regularly on ISS, cleaning procedures can decrease impact on crew
  - Air Contamination – good air quality and filtration can limit likelihood of airborne and droplet-based infections among crew
  - Water Contamination - water quality monitoring and cleaning helps limit infections in crew

  - Pharmaceutical Contamination - repackaged pharmaceuticals are susceptible to contamination increasing risk for infection among crew
  - Food Contamination – inadequate packaging and storage conditions for crew food could lead to infections including gastroenteritis
- Countermeasures that affect microbial levels must be included in the Crew Health and Performance System and accommodated in Vehicle Design. These are affected by the HSIA (Risk) and include:Countermeasures include the storage conditions which if compromised could increase contamination of food and pharmaceuticals; the storage conditions are also impacted by the food system available which is represented in the DAG by the Food and Nutrition (Risk)
  - Preventive Source Control includes monitoring, regular cleaning, filtration and other modes of limiting spread of microbes
  - Hygiene includes personal hygiene such as regular showers, dental hygiene, and other personal cleaning that limits the development of Infection.
  - Environmental Monitoring Capability is necessary to Detect Contamination levels in the air, water, and surfaces. This enables Intervention Source Control measures like cleaning or maintenance of filtration systems.
  - Medical Treatment Capability includes antibiotics, antifungal, and antiviral medications, as well as other supportive care, intended to minimize consequence of infection and prevent the development of sepsis
- Infections and Sepsis affect cognitive function, mood and performance and therefore affect Behavioral (Risk) and Team (Risk) which negatively impacts Individual Readiness and Crew Capability.

Integration with other risks:

Risk of Adverse Health Event Due to Altered Immune Response
Multiple studies have confirmed that the astronaut immune system is dysfunctional with some evidence of increased susceptibility to infection [93-96]. If the immune system is diminished during a long duration mission, the astronaut may become vulnerable to infection with opportunistic or commensal bacteria that would not otherwise be a risk to the crew.

Risk of Adverse Outcomes Due to Inadequate Human Systems Integration Architecture
Vehicle design is critical to limit microbial cross-contamination of the space habitat. High microbial areas with potential for vehicle contamination, including the waste hygiene facility and trash receptacles, should be designed to avoid interaction with areas that could lead to crew infection, such as the galley and crew sleeping area.

Risk of Performance Decrement and Crew Illness Due to Inadequate Food and Nutrition
The preparation and consumption of spaceflight food remains a potential route of infection for astronauts during spaceflight missions. Preflight monitoring of ISS spaceflight foods has indicated the presence of etiological agents of gastroenteritis, including *S.* Typhimurium, and toxin producing organisms, including *S. aureus* and *Aspergillus flavus*, indicating a clear route of infection.

Risk of Adverse Cognitive or Behavioral Conditions and Psychiatric Disorders

Illness caused by infection can directly affect crew behavior and performance, as symptoms of disease (e.g., headaches, fever, diarrhea, inflammation) can interfere with behavior and nominal operational activities. Evidence also indicates a relationship between mood and behavior with the species in the gut microbiome [97].

Risk of Performance and Behavioral Health Decrements Due to Inadequate Cooperation, Coordination, Communication, and Psychosocial Adaptation within a Team
Illness can modify astronaut mood and behavior, creating a risk of inadequate communication and cooperation. In the event of severe illness (*e.g.*, urinary tract infection on Apollo 13), team activities would need to be greatly modified to accommodate the loss of crew time to the infected crewmember and other crewmembers as caregivers.

SECTION IV: KNOWLEDGE BASE

Micro-101: Evaluate the effects of isolation, confinement and weightlessness on changes in the vehicle microbiome, the human microbiome and microbial virulence.

Alterations in microbial responses to spaceflight and spaceflight analogue culture compared to terrestrial responses has been well-documented over the past 55 years [4,5,18,19]. **The evidence clearly indicates alterations in microbial responses that could affect microbial virulence and pathogenesis** [4-7,17,50,98], **although enabling the translation of this information to operations requires further research.**

Current evidence indicates that the vehicle microbiome is not dramatically affected, reflective of a human home [87-89]. This observation is based on the current ISS design, which could change if the vehicle systems are modified (*e.g.*, addition of larger plant growth chambers or biological waste remediation system) or operational protocols (*e.g.*, housekeeping, microbiological remediation approaches) are changed. The knowledge base of spaceflight effects on astronaut microbiomes is limited by the very small sample size and currently prevent any substantial conclusions. In addition, alteration of specific characteristics that could impact habitat sustainability and astronaut health, such as biofilm formation [56,57], still have not been fully elucidated.

Overall, **the ability to modifying the current microbiological spaceflight requirements and/or develop countermeasures to address this aspect of space travel has been greatly limited due to the lack of knowledge about which opportunistic and obligate pathogens are affected primarily due to the lack of understanding about the stimulus/stimuli and mechanism(s) behind these unexpected microbial responses**. While several mechanistic concepts have been proposed [4,18], this gap in knowledge remains a missing foundational element in mitigating this risk.

Current evidence behind the mechanism(s) behind these unexpected microbial responses in spaceflight and spaceflight analogue suggests an association between the evolutionarily conserved global regulator, Hfq, based on the transcription profiles from the *S.* Typhimurium spaceflight studies [6]. This finding was corroborated with post-flight data performed in the RWV [6]. Interestingly, increased virulence corresponding to spaceflight culture was connected with downregulated hfq, which is different from previous terrestrial reports [99], suggesting a novel molecular mechanism for spaceflight cultured bacteria. Corroborating this proposed mechanistic

connection was the discovery that Hfq was also observed in spaceflight and/or spaceflight analogue responses of *P. aeruginosa* [75,98], *S. aureus* [66], and *Vibrio fischeri* [100].

Different studies using the RWV indicated that the response in this environment may be the result of a mechanotransductive stimulus, that had previously been proposed [4]. Early studies of bacteria in the RWV that investigated the production of secondary metabolites by *E. coli* discovered that the addition of fluid shear from a glass bead added to the vessel would change the site of the metabolite accumulation from the media to within the cell [101]. In a later study, progressive addition of fluid shear to the RWV environment incrementally altered phenotypic and gene expression data incrementally toward control responses [102]. The potential of a spaceflight-associated mechanotransductive response, which is the product of changes in physical forces on the cell membrane would not be without precedence, as shear forces have been demonstrated to impact microbial responses [103,104]. Indeed, a number of bacterial cytoskeletal structures, such as MreB (actin homolog) and FtsZ (tubulin homolog) have been identified [105].

Other evidence suggests a wide array of possible contributing factors, including RWV transcriptomic data suggesting decreased oxygen availability [66,75] and spaceflight data showing repression of the enhanced virulence response of S. Typhimurium when high inorganic salts were added to the media [7]. In addition, Kacena *et al*. found that growth on semisolid agar negated changes in enhanced microbial growth noted in liquid cultures, suggesting that a physical artifact from the agar influenced the bacterial response [106]. Collectively, one common factor in all of the information is the absence of any evidence suggesting bacteria and fungi have a direct response to gravity [18]. Rather, the proposed mechanism(s) are likely indirect effects created by the lack of gravity on the microorganism's environment. **Future work should include understanding the factors stimulating unexpected responses of microorganisms in response to spaceflight and spaceflight analogue environments.** Without this information, the translation of data toward the development of countermeasures and the modification of current microbiological requirements will be greatly limited.

Micro-102: Evaluate whether deep-space radiation has an additive or synergistic effect with weightlessness/isolate/confinement on microbial types, numbers and virulence.

While possible, no evidence is available indicating that virulence of a pathogen increases in response to exposure to spaceflight radiation. However, during long-duration spaceflight, astronauts may be exposed to energetic particle radiation, such as protons and heavy ions from solar particle events and galactic cosmic radiation, which may damage epithelial cell tissue and its function [107]. In combination with potential radiation-induced changes in the astronaut microbiome, these epithelial cells may be more susceptible to infection.

The contribution of spaceflight radiation to infection is unclear; however, in combination with other factors, such as increased pathogen virulence [6], dysfunctional immune system [93], and/or lung inflammation due to lunar dust inhalation [108], the risk of infection may be synergistically higher in astronauts during exploration missions beyond low earth orbit. **Future work should include evaluations of the contribution of these spaceflight factors toward increase risk of infectious disease and if their contributions "stack" synergistically to increases the overall risk at a higher rate than would be normally expected.**

Micro-103: Evaluate whether atmospheric composition (for example, elevated CO2 levels) is a significant contributor to changes in the microbial profile of spaceflight.

Multiple stressors in the spaceflight environment have the potential to increase disease risk. One potential stressor is the contribution of atmospheric constituents, such as CO_2 at higher atmospheric concentration than would be observed in terrestrial settings. The current Spaceflight Maximum Allowance Concentration (SMAC) for 24-hour average CO_2 is 0.4% (compared to 0.04% normally found in Earth's atmosphere). These elevated ambient CO_2 levels aboard the ISS could potentially influence the diversity and phenotypic responses of the resident microbial communities from both the spacecraft environment (air, surface, water) and crewmembers. Evidence from studies performed in food microbiology, marine biology and terrestrial environmental biology suggest altered gene expression, selective bacterial inhibition, increased growth and diversity, and increased antibiotic resistance of bacterial communities individually and in biofilm formation when exposed to increased levels of CO_2 [109-111]. While many of these findings are based on levels of CO_2 higher than would be found during spaceflight exploration missions, the potential for subtle changes in CO_2 levels to exacerbate infectious disease risks warrants an evaluation of pathogen responses to spaceflight CO_2 conditions.

Micro-201: Evaluate the contribution of changes in microbial numbers, types and virulence on the likelihood and consequence of adverse health events (infection and allergic response), during the mission.

Infectious disease does occur during spaceflight in spite of rigorous vehicle design and stringent operational protocols to prevent its occurrence [1,2]. However, aligning the incidence of disease with the presence and virulence of microorganisms is challenging, as monitoring efforts are limited to single timepoints that are often one to three months apart. In addition, diagnostic capabilities are limited during spaceflight, preventing conclusive data connecting crew symptomology with microbial agents. **As more monitoring data may not provide a better understanding of this Gap, new data analysis and modeling techniques should be investigated**. These techniques could include machine learning and other advanced data analysis approaches.

Micro-202: Evaluate the contribution of changes in microbial numbers, types and virulence on the likelihood and consequence of non-infection-based effects on health and performance, including: decrease in cognition/mood/performance/blood-brain barrier (BBB) function related to the change in the gut's microbiome and gut-brain axis, increase in cardiovascular health risks, effects of change in gut microbiome on metabolism of nutrients, and correlation with inflammation.

Microorganisms are a normal part of the astronaut microbiome. Accordingly, terrestrial evidence suggests that alterations in the astronaut microbiome could alter their health, performance, and behavior through the gut-brain axis [31,97,112]. Research into the effect of the gut microbiota on the human body covers a wide range of possible relationships including nutrient metabolism [113], inflammation [114], and cardiovascular health [115]. **As evidence on these relationships is rapidly developing, future work should include monitoring developments in the field and collaborating in interdisciplinary studies when appropriate.**

Micro-301: Identify, develop, and implement in-flight microbial monitoring/diagnostic tools for support of research and crew health during Gateway, Lunar and Mars missions.

Since 2000, microbiological monitoring of ISS vehicle air, vehicle interior surfaces, and potable water has provided insight into the safety of the habitat. As spaceflight missions extend beyond low earth orbit, the need for efficient, simple microbial monitoring/diagnostic techniques that enable autonomous decisions by the astronauts will need to be developed. **Future work should include evaluating the progress and capabilities of operationally relevant hardware technologies that are being developed by NASA programs (e.g., Advanced Exploration Systems (AES), SBIR/STTR).**

Micro-401: Test, optimize and validate existing terrestrial or novel technologies that can maintain in-flight microbial counts, types and virulence at terrestrial equivalent levels.

Mitigation technologies of microbial contamination and crew infection have remained relatively unchanged. While these approaches have been successful in controlling mitigating risks to astronaut health, limited resource availability (*e.g.*, upmass, available volume) during long duration missions beyond low earth orbit will prevent many technological approaches, such as the use of individually wrapped disinfection wipes for remediation of contamination events. **Future work should include evaluating the progress and capabilities of operationally relevant preventive agents and countermeasures, such as disinfectants, anti-inflammatories, and antibiotics, that are either commercially available or being developed by NASA programs.**

SECTION V: CONCLUSIONS

Microbial contamination and crew infection continue to occur on spaceflight missions; however, the extent to which spaceflight exacerbates disease risk is not clear. Growth of microorganisms in the spaceflight environment has been shown to increase microbial virulence, although the types of microbes and the cause behind this increase is not fully known. Furthermore, the confined spaces and recycled air and water systems in spacecraft environments in combination with spaceflight specific factors (*e.g.*, diminish immune function, inhalation of celestial dusts, chronic elevated radiation) may increase the overall risk of disease either alone or by contributing synergistically to a larger stacked risk. In addition, microbial characteristics, such as antibiotic resistance and biofilm formation may be altered, limiting countermeasure efficacy and vehicle life support systems. Greater knowledge is required to determine appropriate countermeasures, requirements, and processes for design and monitoring during exploration missions beyond low earth orbit.

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RESOURCES

1. NASA Human Research Roadmap (https://humanresearchroadmap.nasa.gov/Risks/risk.aspx?i=80)
2. NASA Task Book (https://taskbook.nasaprs.com/tbp/welcome.cfm)
3. *Review of NASA's Evidence Reports on Human Health Risks: 2014 Letter Report*. National Academies of Science Engineering, Medicine (https://www.nap.edu/catalog/18983/review-of-nasas-evidence-reports-on-human-health-risks-2014)
4. Microbial Risk Assessment Guideline: Pathogenic Microorganisms with Focus on Food and Water. Report No. FSIS Publication No. USDA/FSIS/2012-001; EPA Publication No. EPA/100/J12/001, (U.S. Department of Agriculture/Food Safety and Inspection Service (USDA/FSIS) and U.S. Environmental Protection Agency (EPA), 2012) (https://www.epa.gov/sites/default/files/2013-09/documents/mra-guideline-final.pdf)
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